Atomic Force Microscopy-based Cell Nanostructure for Ligand-conjugated Quantum Dot Endocytosis

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Abstract While it has been well demonstrated that quantum dots (QDs) play an important role in biological labeling both in vitro and in vivo, there is no report describing the cellular nanostructure basis of receptor-mediated endocytosis. Here, nanostructure evolution responses to the endocytosis of transferrin (Tf)-conjugated QDs were characterized by atomic force microscopy (AFM). AFM-based nanostructure analysis demonstrated that the Tf-conjugated QDs were specifically and tightly bound to the cell receptors and the nanostructure evolution is highly correlated with the cell membrane receptor-mediated transduction. Consistently, confocal microscopic and flow cytometry results have demonstrated the specificity and dynamic property of Tf-QD binding and internalization. We found that the internalization of Tf-QD is linearly related to time. Moreover, while the nanoparticles on the cell membrane increased, the endocytosis was still very active, suggesting that QD nanoparticles did not interfere sterically with the binding and function of receptors. Therefore, ligand-conjugated QDs are potentially useful in biological labeling of cells at a nanometer scale.

Key words quantum dot; endocytosis; nanostructure; atomic force microscopy

Recently, our understanding of cancer diagnosis and biological imaging has been significantly extended by the application of fluorescent semiconductor nanocrystals (quantum dots, QDs) because of their unique optical properties and outstanding biocompatibility [1−13]. It is interesting to note that one of the most important applications of ligand-conjugated QDs is to target them to the cell surface receptors. For example, Chan and Nie [11] showed that QD bio-conjugates were transported into the cells by receptor-mediated endocytosis and detected the clusters and aggregates on the cell surface. Osaki et al. [12] revealed the size effect of endocytosis in the subviral region and demonstrated that the endocytosis is highly size-dependent because the size complementarity governed the molecular recognition in small host-guest systems. Data of Rosenthal et al. [13] suggested that serotonin-labeled nanocrystals (SNACs) interacted with the serotonin transporter protein (SERT) in transfected HeLa cells and oocytes in vitro. They also showed that fluorescent SNACs could be used to visualize SERTs expressed in human epithelial kidney cells in an antidepressant-sensitive manner [13].

Although the potential of QDs in biological labeling and cancer diagnosis has been well described, little is known about the cellular nanostructures response to the QD labeling and receptor-mediated endocytosis of the QD-ligand. It is important to point out that cell surface structures at the nanometer scale may have important roles in shaping the function of the particular molecules on the cell surface. By monitoring the binding, internalization and function of ligand-conjugated QDs at the nanometer scale, ligand-conjugated QDs may help to advance the applications of QDs in targeting drug delivery, protein transportation, ligand-receptor binding and signal trans-
duction in biology and immunology. Furthermore, atomic force microscopy (AFM) has emerged as a powerful tool in cell biology studies at the nanometer scale under near physiological condition [14]. Therefore, AFM visualization and determination of responses in cells at the nanometer scale may elicit particular molecular recognition and offer new insights into the binding and internalization of ligand-conjugated QDs.

In this paper, AFM was used to characterize the cellular nanostructure evolution when HepG2 cells were cultured in ligand-conjugated QDs and free QDs for different time periods. At the same time, confocal laser scanning microscopy images, mean fluorescent intensity (MFI) and percentages of positive cells obtained by flow cytometry were used to reveal the fluorescent characteristics at different stages of endocytosis. As a result, the combined methods of AFM, confocal laser scanning microscopy and flow cytometry demonstrated that transferrin (Tf)-conjugated QDs (Tf-QDs) interact specifically with the Tf receptor (TfR) in human hepatic cancer cells. The internalization of ligand-conjugated QDs during endocytosis appears to be a linear process with time. These experiments suggested that AFM-based nanostructure analysis should provide novel insights into receptor-mediated transductions and fluorescent nanocrystal-based molecule detection.

Materials and Methods

Reagents

Biotin-Tf was purchased from Molecular Probes (Eugene, USA). Streptavidin-conjugated 605 nm Qdots was purchased from Quantum Dot (Hayward, USA). All other reagents were of analytical grade.

Cell culture and ligand-conjugated quantum dot incubation

HepG2 cells were seeded in a 6-well Lab-Tek chamber (Nalge Nunc, Naperville, USA) and grown in RPMI 1640 medium with glutamax supplemented with 10% fetal calf serum in 5% CO₂ at 37 °C. Two activation methods were applied to activate TfR: (1) first, the biotinylated Tf were added directly in cell culture well to activate the TfR on cell surface, and then streptavidin-QDs were loaded into cell culture well to bind Tf; (2) the Tf-QD conjugates were prepared in test tube using the strong binding between streptavidin and biotin and then Tf-QD conjugates were loaded into cell culture well. Tf-QD complexes were formed by incubating biotin-Tf (47 ng/ml) and streptavidin-QDs at 4 °C with mixture for 30 min and added to the cell culture. Cells were centrifuged to remove unbound Tf-QDs and fixed by 4% formaldehyde. Consecutive binding of biotin-Tf and streptavidin-QDs was conducted with cells labeled by 1.9 ng/ml biotin-Tf in culture medium and then incubated with 200 nM QDs for different durations. Then the cells were washed, centrifuged and fixed for imaging.

Microscopy and flow cytometry

Atomic force microscopy was performed in contact mode with a commercial atomic force microscope (AutoProbe CP, Veeco, USA) at room temperature. The AFM images were planar-leveled using the software (Thermomicroscopes Proscan Image Processing Software Version 2.1) provided by the manufacturer. The contact angles were measured by this software directly. Here, the contact angles mean the shrinking degree of cell. The larger contact angels mean the higher degree of cellular shrinkage. Confocal laser scanning microscopy was performed with a Bio-Rad MRC600 microscopy system (Bio-Rad, Hercules, USA) using a 488-nm line excitation of an air-cooled 100 mW argon laser. The emitted fluorescence was detected through the combination of the appropriate AG2 filter set with a high pass at 605 nm. Cell suspension with a density of about 5×10⁵ cells per milliliter was processed for flow cytometry under commercial flow cytometry facility (FACSCalibur; BD Bioscience, Franklin Lakes, USA).

Results

Fine AFM images (Fig. 1) of individual cells without Tf-QD endocytosis at the nanometer scale were readily observable. These images showed the characteristic features of cells with an average diameter of about 16 μm. The cell membrane showed a smooth feature with several holes, which allowed endocytosis of nutrients. The contact angle between the cell and the substrate was about 27°.

Cells were exposed to biotinylated Tf and TfRs were activated to bind streptavidin-QDs, allowing specific binding of biotin-Tf and streptavidin-QDs. After exposing to streptavidin-QDs for about 1 min, the filopodia of cells became larger (Fig. 2) probably due to binding of QDs to Tf. This result clearly demonstrated that Tf activated the TfR within 1 min. It should be noted that Lidke et al. [4] also reported similar results using confocal laser scanning microscopy, suggesting that the QDs bind to the cell filopodia. Importantly, this consistency revealed the high
sensitivity and reliability of AFM-based nanostructure analysis. After about 15-min incubation, the cell edge tended to shrink and the cell body expanded significantly, suggesting that rapid and extensive endocytosis of Tf-QD occurred. Collectively, these results demonstrated that the nanoparticles activate the cell receptors readily and do not damage the cellular receptor activity and the endocytic pathways. Also, the changes in cell nanostructures at different time points suggested that endocytosis might be a dynamic process.

To determine whether the receptor activation pathway was involved in endocytosis at the nanometer scale, we activated the cell receptors using an alternative approach. We preloaded the streptavidin-QDs with biotin-Tf to obtain a Tf-QD complex. Importantly, after culturing the cells in Tf-QDs for 15 min, the cell body expanded and the cell edge shrank, supporting the feasibility of the idea that ligand-conjugate QDs preloading and the desired receptor-mediated transportation of ligand-conjugate QDs could be achieved by such strategy as well. Interestingly, these two different receptor activation pathways gave similar results on cellular nanostructure (Figs. 3 and 4). It should be noted that bulges could be observed on the cell edge and these bulges may be cell vesicles or nanocrystals of Tf-QDs.

There are at least two differences between control cells and cells that have undergone endocytosis of ligand-conjugated QDs. First, cells with QDs endocytosis showed expansive body and contractive edge. As a result, the shrinkage has led to an increase in the contact angles between the edge and substrate. Second, the cell surface has provided more space to allow more ligand-conjugated QDs to bind onto the cell membrane, which have resulted in a dramatic increase of nanoparticles on the cell surface.

Fig. 1  Atomic force microscopy image of a HepG2 cell (A) and its typical profile along the dashed line (B) without exposure to transferrin-quantum dots
The contact angle is about 27º. The white arrow indicates the blank filopodia. θ represents the contact angle between cell edge and substrate.

Fig. 2  Atomic force microscopy image of a HepG2 cell activated by biotin-transferrin (Tf) and then exposed to streptavidin-quantum dots (QDs) for 1 min
The white arrow indicates the filopodia binding Tf-QDs.

Fig. 3  Atomic force microscopy image of a HepG2 cell activated by transferrin and then exposed to quantum dots for 15 min (A) and typical cell profile and contact angle (B)
A typical cell profile shows the height (about 5 μm) and contact angle (about 60º) increasing dramatically between cell and substrate. Based on the profile line, it is clear that the cell expands dramatically. θ represents the angle between cell edge and substrate.
The differences in size and shape between these cells could be attributed to the following factors. First, the binding and internalization of ligand-conjugated QDs resulted in the expanse of cell body. Second, the non-specific endocytosis Tf-QD and aggregation property of QDs after leveling the storage buffer condition may also result in an increase in the nanoclusters on the cell surface.

In order to confirm that the differences were the results of the receptor-mediated Tf-QD endocytosis rather than unspecific QD binding, we cultured the cells in the presence of free QDs. As expected, the cell membrane remained almost intact and relatively unaffected although there were some particles on the cell surface (Fig. 6). It should be pointed out that such particles [Fig. 6(B)] were likely the results of nonspecific binding and aggregation. This result was further confirmed by flow cytometry (Fig. 7). Compared with the blank cells, there is no significant increase in MFI, but the significant positive cell percentage could be observed in cells exposed to free QDs. The background fluorescence of cells exposed to free QDs obtained by flow cytometry (Fig.7) should arise from nonspecific binding of QDs during cell fixation and the cell autofluorescence. In contrast, after the cells were exposed to Tf-QDs, both MFI and positive cell percentage increased simultaneously as the exposure time increased. Taken together, these results, again, illustrate that Tf-QD is highly

Fig. 4 Atomic force microscopy images of a HepG2 cell exposed to transferrin-quantum dots for 15 min
The white arrows indicate the vesicles induced by endocytosis.

Fig. 5 Atomic force microscopy images of HepG2 cells exposed to transferrin-quantum dot (QD) for 150 min (A) and 180 min (B), respectively
QDs and Tf concentration are adapted to 200 nM and 47 ng/ml, respectively.

Fig. 6 Atomic force microscopy images of a HepG2 cell exposed to free quantum dot (QD) for 30 min (A) and the non-specific binding of transferrin-QDs (B)
Most parts of the cell surface retain smooth. The white arrow indicates nonspecific binding and aggregation of ligand-conjugated QDs.

Fig. 7 Mean fluorescent intensity and percentage of positive cells obtained by flow cytometry
0, the control cells; 1, the cells after exposure to free QDs for about 30 min; 2, the cells with exposure to transferrin (Tf)-quantum dot (QD) for about 30 min; 3, the cells with exposure to Tf-QD for about 168 min. Positive cells are the fluorescent cells. MFI1, mean fluorescent intensity for positive cells; MFI2, mean fluorescent intensity for all cells.
specific and potentially useful in activating and binding Tf to cell receptors.

The inherent stability of the biotin-Tf and streptavidin-QDs makes us able to obtain the desired degree of ligand loading simply by adjusting the mixing stoichiometry. To further investigate the effect of loading rate on nanostructure response for endocytosis of ligand-conjugated QDs, we cultured the cells after exposure with different loading rates of biotin-Tf and streptavidin-QDs (Fig. 5). Again, cells showed the characteristic features of an expansive body and contractive edge (Fig. 5). Furthermore, increases in Tf-QD concentration and exposure duration resulted in more accumulation of Tf-QD on the cell surface (Fig. 5), demonstrating that exposure duration enhanced the binding of the ligand-conjugated QDs before internalization. Moreover, the nanoparticles on the cell membrane increased while the endocytosis was still very active. Taken together, these evidences suggested that the QD nanoparticles did not interfere sterically with the binding and function of receptors. Therefore ligand-conjugated QDs are potentially useful in biological labeling of cells at the nanometer scale.

Based on the AFM images, the fluorescent signal change of Tf-QD in cell bodies might be a dynamic process coupled with binding and internalization of ligand-conjugated QDs. This inspires us to further examine whether the fluorescent signal might correlate with the cellular nanostructure evolution. In Fig. 8, three representative

Fig. 8 Confocal laser scanning microscopy images of cells exposed to Tf-QD and the fluorescent intensity corresponding (A–C) For 106 min, 149 min and 168 min, respectively. (D–F) The fluorescent intensity as the function at the location of the dashed line in (A–C), respectively. Magnification, 400×. ©Institute of Biochemistry and Cell Biology, SIBS, CAS
fluorescent images of cells, after 106 min, 149 min and 168 min of exposure to Tf-QDs are shown. In these samples, the fluorescent intensity is enhanced dynamically. Furthermore, the fluorescent intensity in the central part of the cell is significantly stronger than that in the cell edge. This became clearer as the exposure time was increased. The most likely explanation for this phenomenon is the internalization of Tf-QDs upon the binding of Tf-QDs to cell Tf receptor. Consistently, AFM observations also suggested the similar phenomenon, suggesting the reliability and applicability of AFM-based nanostructure analysis. We also found that internalization of Tf-QD appears to be linearly related to time (Fig. 9). Importantly, this observation is consistent with the results of EGF-QDs [4], whose internalization is also related to time linearly.

Based on our observations of cellular characteristics of endocytosis of Tf-QDs, we proposed a model (Fig. 10) for the endocytosis of QDs. It seems that the endocytosis of Tf-QDs represents a typical trajectory of ligand-conjugated QDs activating cell receptors, binding onto cell surface, and internalizing. As illustrated above, before the internalization of Tf-QDs, not only Tf but also Tf-QD conjugates were able to activate Tfr, therefore, two different pathways could achieve stage one. For stage two, ligand-conjugated QDs bind to the cell receptor. Therefore, the nanoclusters on the cell surface increased dramatically. For stage three, the ligand-conjugated QDs experienced internalization, resulting in the expansion of the cell body.

While QDs have been widely used as a novel fluorescence probe for both in vivo and in vitro studies, the desired cellular nanostructure responses for the biological label of QDs have been poorly elucidated. The well-defined cellular nanostructure analysis should certainly be an important step for the development of better biological labeling of QDs. First, elucidation of cellular nanostructures by direct AFM visualization of QDs endocytosis should significantly extend our understanding of cell characteristics and be useful for designing and interpreting molecular probes at the nanometer scale. Second, the insights provided by AFM-based nanostructure characterization at the nanometer scale allow us to be in a unique position to study the endocytosis and nanoparticle targeted delivery, which are difficult to be achieved using other methods such as TEM and SEM because they require complex pretreatments, which usually damage the cell structure and functional molecules on the cell membrane. Third, at the nanometer scale, spatial prevention and nonspecific interactions make it difficult to detect the desired molecules on the complex cell surface directly. Apparently, QDs fluorescent probes and AFM can be used to study the inside or surface molecules of cells, receptor-targeted cross membrane transportation, and the virus infection at the nanometer scale. Together with confocal laser scanning microscopy...
and flow cytometry, direct AFM visualization provides the background and principles of cellular nanostructure analysis, making a case for the use of such measurements as disease markers at the nanometer level.

While many groups have studied the basic principle and application of QDs in biological labeling in in vivo and in vitro studies, little is known about the nanostructure evolution induced by endocytosis of ligand-conjugated QDs. Our AFM study shows that ligand-conjugated QDs are highly specific and bind strongly to the receptor. Also, the TfR on cell surfaces can be activated either by Tf or Tf-QD. After receptor stimulation, both pathways can achieve the desired endocytosis. In this study, we found that the internalization of Tf-QD is linearly related to time. AFM direct visualization of ligand-conjugated QDs endocytosis provides novel insights into the biological labeling of QDs, which should be useful for the design and application of QDs fluorescent probes.

References


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